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Use of fluorescence resonance energy transfer to analyze oligomerization of G-protein-coupled receptors expressed in yeast

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Abstract

Oligomerization or dimerization of G-protein-coupled receptors (GPCRs) has emerged as an important theme in signal transduction. This concept has recently gained widespread interest due to the application of direct and noninvasive biophysical techniques such as fluorescence resonance energy transfer (FRET), which have shown unequivocally that several types of GPCR can form dimers or oligomers in living cells. Current challenges are to determine which GPCRs can self-associate and/or interact with other GPCRs, to define the molecular principles that govern these specific interactions, and to establish which aspects of GPCR function require oligomerization. Although these questions ultimately must be addressed by using GPCRs expressed endogenously in their native cell types, analysis of GPCR oligomerization in heterologous expression systems will be useful to survey which GPCRs can interact, to conduct structure–function studies, and to identify peptides or small molecules that disrupt GPCR oligomerization and function. Here, we describe methods employing scanning fluorometry to detect FRET between GPCRs tagged with enhanced cyan and yellow fluorescent proteins (CFP and YFP) in living yeast cells. This approach provides a powerful means to analyze oligomerization of a variety of GPCRs that can be expressed in yeast, such as adrenergic, adenosine, C5a, muscarinic acetylcholine, vasopressin, opioid, and somatostatin receptors.

Keywords: G-protein-coupled receptor; Oligomerization; Dimerization; Fluorescence resonance energy transfer; Scanning fluorometry; Cyan fluorescent protein; Yellow fluorescent protein; Yeast

1. Introduction

Evidence accumulated over the past three decades has suggested that G-protein-coupled receptors may exist as dimers and/or oligomers. However, widespread appreciation of this concept has come only recently. This has occurred because direct and noninvasive biophysical techniques such as fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) have shown that a variety of GPCRs can form homo- and/or heterooligomeric assemblies in living cells.

Several studies have begun to indicate that oligomerization is required for several aspects of GPCR function. For example, dimerization of peptides corresponding to the third cytoplasmic loops of certain GPCRs increases their potency as G-protein activators in vitro [1], and peptides that interfere with receptor dimerization in vitro can inhibit signaling by β_2-adrenergic receptors [2]. Furthermore, overexpression of dominant-negative receptor mutants can inhibit oligomerization of wild-type receptors [3]. Finally, coexpression of receptors can modify their function, as shown by studies of heterodimerization between $GABA_B$-R1 and R2 [4–6], κ and δ opioid [7], m2 and m3 muscarinic [8], and $5\text{-}HT_{1B}$ and $5\text{-}HT_{1D}$ serotonin [9] receptors, and non-subtype-specific heterodimerization between adenosine/dopamine [10] and opioid/adrenergic receptors [11]. Therefore, it seems likely that the function of many GPCRs will be determined by their ability to self-associate and/or interact with other GPCRs. Defining which receptors interact with themselves and each other, establishing the molecular principles that

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govern these specific interactions, and determining the functions of specific GPCR complexes are therefore important goals of the field.

2. FRET theory and practice

FRET provides a powerful means to study GPCR oligomerization. Unlike biochemical approaches such as coimmunoprecipitation which require receptors to be solubilized from membranes with detergent, a process that often results in inactivation of a GPCR, FRET can detect interaction between functional receptors in living cells or plasma membrane fractions. FRET can occur between molecules separated by <100 Å [12]. This property makes FRET an ideal means of analyzing GPCR oligomerization because the predicted center-to-center distance between GPCRs in a dimer/oligomer is ~40 Å, as indicated by the dimensions of a rhodopsin monomer [13,14].

FRET between GPCRs could be investigated by using various types of fluorescence donor–acceptor pairs. The GFP variants cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), developed for FRET by Tsien and co-workers [15–17] have spectral properties that have made them, from our experience, preferable to the blue fluorescent protein (BFP)–green fluorescent protein (GFP) FRET pair. Tsien and colleagues showed that FRET occurs between CFP ($\lambda_{max(abs)} = 434$ nm, $\lambda_{max(emis)} = 476$ nm) and YFP ($\lambda_{max(abs)} = 514$ nm, $\lambda_{max(emis)} = 527$ nm) when these proteins are stably associated in close proximity [18]. Like GFP itself, CFP or YFP can be fused to the cytoplasmic domains of GPCRs, often resulting in a functional fusion protein. Furthermore, when CFP and YFP are expressed at typical levels in yeast (~5000–10,000 molecules/cell) their quantum yields are sufficient to detect emission above the fluorescence background of the yeast cell (see below). In contrast, FRET experiments that employ other means of fluorescent labeling, such as with fluorescently labeled antibodies, are not possible because the yeast cell wall excludes peptides larger than about 5 kDa. A schematic depicting FRET between C-terminally truncated GPCRs tagged with CFP and YFP is shown in Fig. 1.

Efficient energy transfer depends on the spectral properties, dipole orientation, and distance between the fluorescence donor and acceptor. Energy transfer requires that the emission spectrum of the donor has significant overlap with the excitation spectrum of the acceptor. In contrast, the excitation spectrum of the donor should have little overlap with the excitation spectrum of the acceptor to minimize direct excitation of the acceptor. Ideally the emission spectra of the donor and acceptor should be completely resolved, but in practice they usually overlap partially. Accordingly, in a typical FRET experiment, the observed fluorescence emission spectrum is the sum of donor emission, acceptor emission due to direct excitation, and acceptor emission due to FRET. Quantification of acceptor emission due to FRET therefore requires precise determination of the proportion of the total emission signal that is due to direct excitation of the acceptor and to overlap of donor and acceptor emission spectra.

Energy transfer efficiency is strongly affected by interfluorophore distance and orientation. Therefore, even large changes in FRET efficiency, as may occur on binding of agonist or antagonist to a receptor, must be interpreted with caution. The efficiency of energy transfer (E) is inversely proportional to the distance separating the donor and acceptor (R) raised to the sixth power, as given by

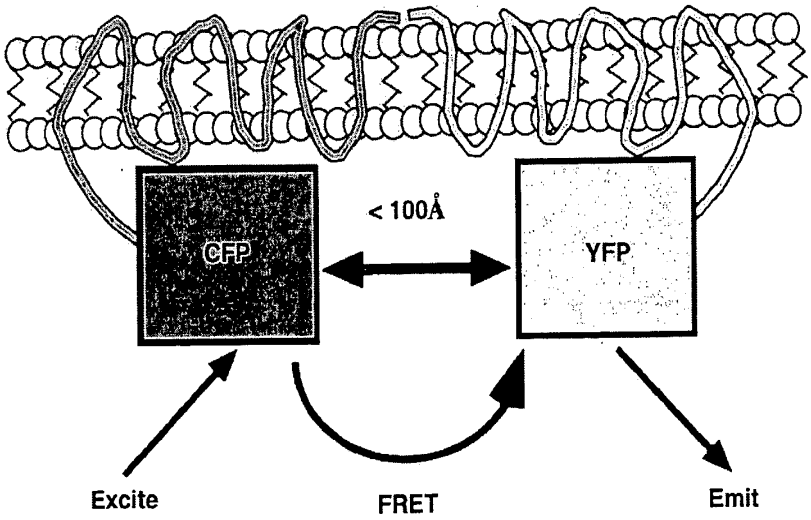

Fig. 1. FRET between CFP- and YFP-tagged GPCRs. Oligomerization of CFP- and YFP-tagged receptors can result in FRET if the fluorphores are in close proximity (<100 Å; see text for details).

$$E = 1/[1 + (R/R_0)^6], \quad (1)$$

where R_0 is the Förster distance at which FRET efficiency is 50%. As a consequence, interfluorophore separation of $1/2R_0$ can yield nearly quantitative energy transfer, whereas separation of $2R_0$ would result in nearly undetectable energy transfer. R_0 is determined by the relative orientation of the donor and acceptor dipoles (k^2), the donor quantum yield (O_D), the spectral overlap between the donor and acceptor (J_{DA}), the refractive index of the medium (n), and Avogadro's number (N) according to

$$R_0^6 = 9000(\ln 10)k^2 Q_D J_{DA}/128\pi^5 n^4 N. \quad (2)$$

Because R_0 of the CFP–YFP donor–acceptor pair commonly used for FRET is ~50 Å, the interfluorophore distance over which FRET can be detected is ~25–100 Å [18].

Changes in the orientation of donor and acceptor fluorophores can also significantly affect FRET efficiency. Whereas the orientation factor (k^2) for dipole–dipole coupling is usually calculated by assuming equal distribution of the fluorophores in all orientations in the excited state, orientation of fluorophores attached to a GPCR or other protein could be restricted in a way that either favors or disfavors efficient FRET. Therefore, a conformational change of a GPCR that increases interfluorophore distance and/or results in disfavorable dipole orientation could cause a large decrease in FRET efficiency without indicating loss of oligomerization per se. Likewise, an increase in FRET efficiency may not necessarily indicate increased efficiency of oligomerization.

Calculation of energy transfer efficiency often involves measurement of the relative fluorescence intensity (F) or lifetime (τ) of the donor in the absence (D) and presence (DA) of the acceptor, according to

$$E = 1 - (F_{DA}/F_D) = 1 - (\tau_{DA}/\tau_A). \quad (3)$$

However, interpreting the results of such calculations in terms of absolute interfluorophore distance can prove difficult for the reasons discussed above. An approximate or *apparent* FRET efficiency can be determined by calculating the ratio of the FRET signal to the signal observed on direct excitation of the acceptor, as discussed in more detail below. When distance calculations are not required, the *apparent* FRET efficiency provides a suitable means to quantify the extent of FRET between tagged molecules.

3. Expression of GPCRs in yeast

The utility of analyzing GPCR oligomerization by performing FRET experiments with yeast is based on an extensive literature that shows various approaches can be used successfully to express functional mammalian GPCRs in the yeast *Saccharomyces cerevisiae* [19–29]. The advantages of yeast expression are cost, speed, and maintenance of receptor pharmacology. Furthermore, the ability to express CFP- and YFP-tagged receptors at equivalent levels is of particular relevance for FRET experiments. This is accomplished by expressing CFP- and YFP-tagged receptors independently from two plasmids that use the same promoter and transcriptional terminator elements, but that have different selectable markers (e.g., *TRP1* and *URA3*) that allow them to be introduced and maintained together in the same cell. Use of single-copy plasmids (*CEN* plasmids, which contain a centromere) provides highly reproducible expression levels because plasmid copy number does not vary significantly. High-copy plasmids (2μ-based) are used to express receptors that do not express well in yeast. However, because the copy number of these plasmids can vary among transformants, the investigator must determine the expression level that has been achieved, as is discussed below.

4. Design of tagged receptors

As an example demonstrating the utility of FRET to analyze GPCR oligomerization in yeast, we have used a GPCR expressed endogenously by yeast cells, the α-factor receptor (*STE2* gene product), to show that GPCRs form oligomers in living cells [3]. This receptor, which is coupled to a yeast G-protein similar to G_i has served as a workhorse for studies of GPCR structure, function, and regulation. We have previously shown that GFP tagging the α-factor receptor at the end of its cytoplasmic C-terminal domain or eight amino acids after the seventh transmembrane domain (position 304) preserves the ability of the receptor to bind agonist with normal affinity and transduce a signal [30]. These C-terminal truncation mutants are, however, defective in receptor phosphorylation, ubiquitination, desensitization, and endocytosis [31]. Therefore, we have used C-terminally truncated receptors (at position 304) tagged with CFP and YFP to ensure that FRET would not detect interactions between desensitized or internalized receptors. Furthermore, preliminary experiments have indicated that FRET is not detected between full-length receptors tagged with CFP and YFP, indicating a dependence on interfluorophore distance, orientation, and/or mobility (data not shown). For other GPCRs it may be necessary to analyze several fusions in which CFP and YFP have been appended at various points within the cytoplasmic C-terminal domain or possibly within intracellular loops if they are sufficiently large. Regardless of the fusion strategy employed, the function and expression of the receptor must be preserved as indicated by

efficiency of cell surface expression and maintenance of affinity and receptor pharmacology.

5. Gene expression and culture conditions

We have expressed α-factor receptor–CFP and receptor–YFP fusions at levels equivalent to endogenous receptors by using the receptor's normal promoter and single-copy plasmids with different selectable markers. However, for mammalian receptors that are expressed inefficiently in yeast, it may be necessary to use strong constitutive promoters, such as *PGK* and *ADH*, and high-copy plasmids. To avoid inhibition of FRET by endogenous untagged α-factor receptors, we have expressed tagged receptors in yeast cells that lack a functional α-factor receptor structural gene on the chromosome (*ste2*Δ mutant). Presumably this would be unnecessary for experiments in which FRET is being detected between tagged mammalian receptors expressed in yeast.

Cell growth conditions have proved to be important for obtaining efficient expression of α-factor receptor fusion proteins. The reason for this is not clear, but poor expression resulting from suboptimal growth conditions is not due to increased endocytosis because the tailless receptor–CFP and receptor–YFP fusion proteins we have used are endocytosis-defective. Regardless of the reason, we use cells within 2–5 days of colony formation following the introduction of plasmids by standard lithium-acetate transformation procedures. Colonies are then used to inoculate 50 ml of synthetic medium lacking appropriate nutrients to select for plasmid-containing cells. Cells are grown with vigorous aeration until reaching midlog phase ($OD_{600} = 0.5$). Cells are then harvested by low-speed centrifugation, washed twice with 25 mM Tris, pH 6.8 (FRET buffer), and suspended in ~5 ml of FRET buffer such that all samples have the same concentration of cells as determined by OD_{600}. Cells are then diluted approximately 1:3 in FRET buffer, resulting in a cell suspension that is slightly turbid, and analyzed by scanning fluorometry with a 3-ml glass cuvette.

6. Equipment

Most modern scanning fluorometers should be sufficient to detect FRET between GPCRs experessed in yeast. We used a Fluorolog-3 spectrofluorometer from the Jobin Yvon/Spex division of Instruments S.A. The excitation and emission slits were set to a 4.0-nm bandpass, high voltage to 950 V, and the shutter mode to auto open. Data were processed using Datamax for Windows Version 2.1 and further analyzed using Excel spreadsheets.

7. Verifying the spectral properties of CFP and YFP expressed in yeast

Fluorescence emission spectra have been used to verify that α-factor receptor–CFP and –YFP fusions exhibit the required spectral properties for FRET experiments in yeast. Illumination of control cells that do not express a CFP or YFP fusion (*cells* in Fig. 2A) at the λ_{max} for excitation of CFP results in detection of cell autofluorescence. Subtraction of the autofluorescence emission spectrum from the emission spectrum obtained following excitation of an equivalent number of cells expressing receptor–CFP (*cells + CFP* in Fig. 2A) yields an emission spectrum identical to that expected for CFP (*CFP* in Fig. 2A). Likewise, illumination of control cells at the λ_{max} for excitation of YFP results in background fluorescence emission, which is subtracted from the emission spectrum obtained with cells expressing YFP-tagged receptors to yield the expected YFP fluorescence emission curve (Fig. 2B). Similar experiments show that illumination at the λ_{max} for excitation of CFP results in low but detectable direct excitation of YFP-tagged receptors (Fig. 2C), and that illumination at the λ_{max} for excitation of YFP does not directly excite CFP-tagged receptors (Fig. 2D).

8. Optimization of the donor excitation wavelength

Because the wavelength typically used to excite CFP results in significant direct excitation of YFP, we have used the following procedure to optimize the donor excitation wavelength such that direct excitation of YFP is minimized. Cells expressing only YFP-tagged receptors are irradiated at a variety of wavelengths near the λ_{max} for CFP excitation, and emission is recorded from 450 to 610 nm to detect YFP fluorescence (Fig. 3). The results indicate that YFP fluorescence emission reaches a minimum at an excitation wavelength of 425 nm. Subsequent experiments have indicated that CFP fluorescence emission is quite robust when an excitation wavelength of 425 nm is used (data not shown). In accord with these results, Table 1 shows the excitation wavelengths and the range of emission wavelengths that have been used subsequently for FRET studies.

9. Optimization of donor and acceptor expression levels

The relative stoichiometry of CFP- and YFP-tagged receptors may adversely affect the efficiency of FRET. Expression of CFP-tagged receptors in excess over YFP-tagged receptors could mask detection of FRET because the CFP emission spectrum overlaps, and therefore could obscure, the YFP emission spectrum. Furthermore, nonstoichiometric expression of CFP- to

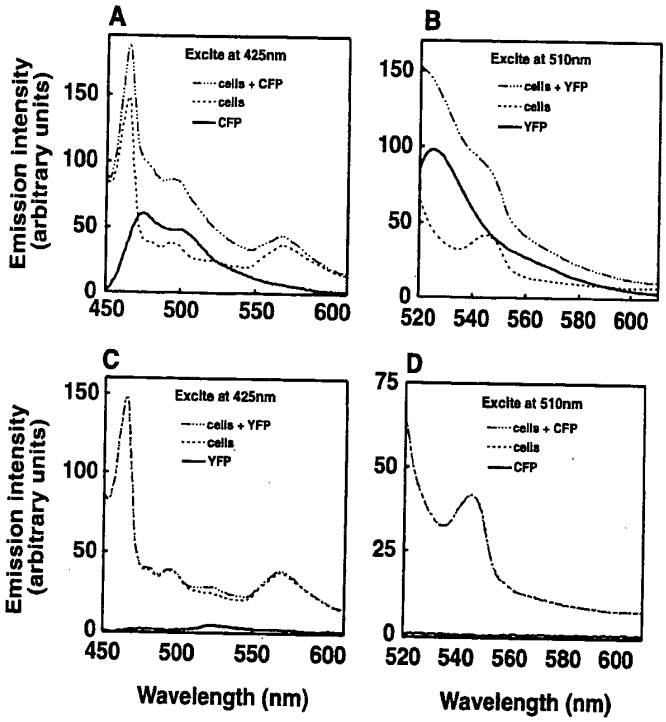

Fig. 2. Fluorescence emission spectra of yeast cells expressing a yeast G-protein-coupled receptor (α-factor receptor) tagged with either CFP or YFP. Yeast cells expressed tailless α-factor receptors fused to CFP (*cells + CFP*), YFP (*cells + YFP*), or empty vector control (*cells*). Washed cells were analyzed by scanning fluorescence emission spectroscopy. Cells were irradiated at 425 nm to excite CFP and scanned for fluorescence emission from 450 to 610 nm (A, C). Cells were also irradiated at 510 nm to excite YFP and scanned for emission from 520 to 610 nm (B, D). The resulting emission spectra of cells expressing either CFP- or YFP-tagged receptors were subtracted from the emission spectra of cells containing empty vector control to generate the emission spectra due only to excitation of the fluorophore.

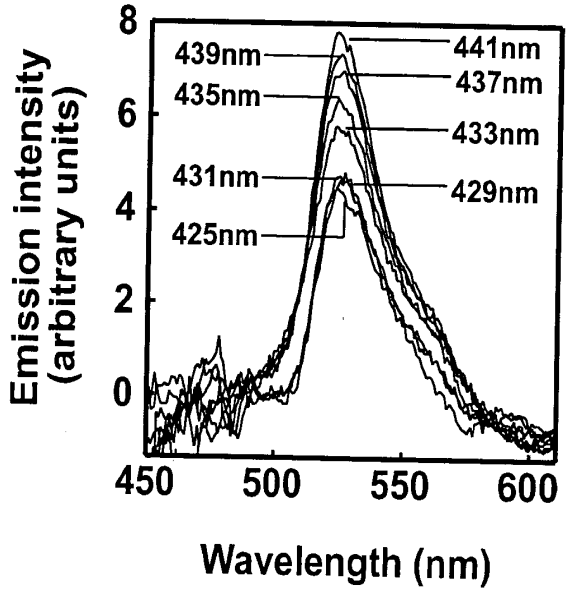

Fig. 3. Wavelength optimization for CFP excitation. Cells that expressed only YFP-tagged receptors or did not express a tagged receptor were irradiated at the wavelengths indicated on each curve. The resultant emission spectra were recorded, and autofluorescence (emission obtained from control cells) was subtracted to yield the YFP emission spectra shown. YFP emission was reduced on excitation at 425 nm, a wavelength that was able to efficiently excite CFP.

Table 1
Excitation and emission wavelengths used for FRET experiments[a]

Fluorophore	$\lambda_{excitation}$ (nm)	$\lambda_{emission}$ scan (nm)
CFP	425	450–610
YFP	510	520–610

[a] For detection of YFP fluorescence, cells were excited at 510 nm and emission was recorded between 520 and 610 nm. For detection of CFP fluorescence, cells were excited at 425 nm and emission was recorded between 450 and 610 nm.

YFP-tagged receptors presumably would result in a decreased number of heterooligomeric receptors capable of yielding a FRET signal. Indeed, by analyzing yeast cells expressing various levels of CFP- versus YFP-tagged α-factor receptors, we have found that an optimal FRET signal occurs with a 1:1 to 1:2 expression ratio (CFP: YFP; data not shown). The ratio of expression can be determined based on the quantum efficiency of CFP and YFP. Using such an approach, we have found that a 1:1 ratio occurs when the peak height of the emission spectrum obtained on direct excitation of YFP at 510 nm is approximately twofold higher than the peak height of the emission spectrum obtained on direct excitation of CFP at 425 nm. Note, however, that this method underestimates CFP emission peak height, be-

cause CFP emission is decreased due to FRET in the presence of YFP acceptor. Nevertheless, we have found that this is not of critical importance because the apparent efficiency of FRET, as calculated below, does not vary substantially over expression ratios of CFP:YFP from 1:1 to 1:2.

10. Data acquisition and analysis for FRET experiments

In each FRET experiment, fluorescence emission spectra are recorded from four samples that contain the same number of cells: (1) control cells that do not express tagged receptors; (2) cells that express only CFP-tagged receptors; (3) cells that express only YFP-tagged receptors; and (4) cells that coexpress CFP- and YFP-tagged receptors. Equivalent cell number is achieved by adjusting cell density to obtain equal fluorescence emission at 450 nm on excitation at 425 nm. This procedure can be used because emission at 450 nm is due solely to cell autofluorescence and is not the result of CFP or YFP emission.

Fluorescence emission due to FRET between CFP- and YFP-tagged receptors is detected by employing the following three-step procedure. Briefly, the procedure involves irradiation of cells at the optimized λ_{max} for excitation of CFP, recording emission spectra, and subtracting the components of the emission spectra due to cell autofluorescence, CFP emission, and YFP emission due to direct excitation; this results in the YFP emission spectrum due only to FRET.

11. Data acquisition and correction for cell autofluorescence

Control cells and cells that coexpress CFP- and YFP-tagged receptors are irradiated at the optimized λ_{max} for excitation of CFP (425 nm) that gives reduced direct excitation of YFP. The autofluorescence emission spectrum obtained from control cells (no tagged receptors expressed) is subtracted from that obtained with an equivalent number of cells coexpressing CFP- and YFP-tagged receptors, resulting in the CFP + YFP emission curve shown in Fig. 4A. This CFP + YFP emission spectrum is a composite of CFP emission, YFP emission due to direct excitation, and YFP emission due to FRET.

12. Subtraction of CFP emission from the CFP + YFP emission spectrum

Cells expressing only CFP-tagged receptors are irradiated at the optimized λ_{max} for excitation of CFP (425 nm). This spectrum (*CFP* in Fig. 4) is normalized to give a CFP emission peak value identical to the CFP emission peak value of the CFP + YFP spectrum obtained from the preceding step. Note that the CFP + YFP spectrum should not be normalized because this would lead to an incorrect determination of apparent FRET efficiency. After normalization, the CFP spectrum is subtracted from the CFP + YFP emission spectrum. This results in a YFP emission spectrum composed of a FRET component and another component due to direct excitation of YFP.

13. Obtaining the YFP emission spectrum due to FRET

To obtain the YFP emission spectrum due only to FRET, it is necessary to subtract the component of the total YFP emission that is due to direct excitation of YFP. This is accomplished as follows. Initially, the YFP emission spectra of cells coexpressing CFP- and YFP-tagged receptors versus cells expressing only YFP-

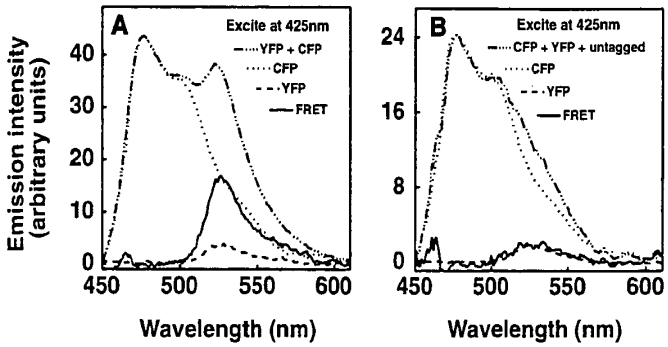

Fig. 4. Use of FRET to detect oligomerization of α-factor receptors in vivo. Yeast cells expressed either no receptors, only CFP-tagged α-factor receptors, only YFP-tagged α-factor receptors, or coexpressed CFP- and YFP-tagged α-factor receptors in the absence (A) or presence (B) of overexpressed untagged α-factor receptors. Cells were irradiated at 425 nm and fluorescence emission was recorded from 450 to 610 nm. Sensitized emission from YFP due to FRET (*FRET*) was calculated as: (*CFP* + *YFP* emission) − (*CFP* only emission) − (*YFP* only emission) (see text for details). The results shown are the averages of six independent experiments.

tagged receptors are normalized for differences in YFP expression level by irradiating these two types of cells at the λ_{max} for YFP (510 nm) and recording their respective YFP emission spectra. Because CFP is not excited at this wavelength, these emission spectra quantify only the level of YFP-tagged receptors. The ratio of the YFP emission peak heights of these two spectra can then be used as a scaling factor to normalize the emission spectrum obtained when cells expressing YFP-tagged receptors are irradiated at the λ_{max} for CFP (425 nm). This normalized emission spectrum (*YFP* in Fig. 4) is then subtracted from the total YFP emission spectrum obtained in step 2 from cells coexpressing CFP- and YFP-tagged receptors. The result is a YFP emission spectrum due solely to FRET (*FRET* in Fig. 4).

14. Calculation of apparent FRET efficiency

As discussed previously, FRET efficiency is usually calculated from the change in the magnitude or lifetime of donor emission in the presence versus absence of acceptor. This requires the ability to quantitatively photobleach the acceptor and measure the resultant change in donor fluorescence. However, quantitative photobleaching of the relatively large (3 ml) samples used for FRET would require lasers that are unavailable to many researchers. We therefore developed a simple alternative that provides a measure of the *apparent* efficiency of FRET. While this alternative is useful for determining the relative ability of GPCRs to engage in FRET, it does not measure the precise efficiency of FRET and therefore is not suitable for distance calculations.

To calculate the apparent efficiency of FRET, we use two spectra obtained during the process of generating the FRET emission spectrum: the YFP emission spectrum due specifically to FRET, and the YFP emission spectrum obtained by irradiating cells coexpressing CFP- and YFP-tagged receptors at the λ_{max} for YFP (Fig. 5). FRET efficiency is calculated by dividing the integrated area of the FRET spectrum by the integrated area of the YFP emission spectrum obtained by excitation at the λ_{max} for YFP. If receptors are dimers, then the theoretical upper limit would be an apparent FRET efficiency of ~50%, because only one-half of the receptor dimers would be composed of a CFP- and a YFP-tagged receptor. However, even in this case, the maximal efficiency would be less because the fluorescent chromophores of CFP and YFP are ~15 Å from the surface of the protein, resulting in a minimum separation of at least 30 Å. In contrast, if receptors form higher-order oligomers, then the number of receptors engaged in productive FRET complexes could approach 100% and thus increase the upper limit of apparent FRET efficiency. Using the methods described above, we have found that the apparent efficiency of FRET between tagged tailless α-factor receptors is 10–15%.

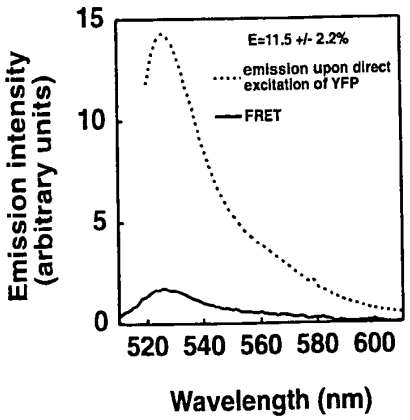

Fig. 5. Apparent efficiency of FRET. Apparent FRET efficiency was calculated as: (integrated FRET curve)/(integrated emission curve obtained on direct excitation of YFP at 425 nm). Efficiency of FRET between CFP- and YFP-tagged α-factor receptors was determined to be 11.5 ± 2.2% as determined from the average of six independent FRET experiments.

15. FRET with control proteins and competition by untagged receptors

If the results of FRET experiments indicate that a GPCR can self-associate, it is important to show that the FRET signal is the result of a stable, specific interaction. This can be demonstrated by showing that FRET does not occur between tagged forms of a given GPCR and another, unrelated GPCR or other type of membrane protein. Furthermore, overexpression of an untagged form of the GPCR itself (but not another GPCR or other type of membrane protein) inhibits FRET between tagged receptors. These experiments are simple to perform in yeast because expression of three or more proteins is easily accomplished through the use of expression plasmids that contain different selectable markers. Furthermore, the methods involved are identical to those used to detect self-association of a given receptor by FRET.

To determine the specificity with which yeast α-factor receptors interact, we have performed FRET experiments and competition experiments using another yeast GPCR (Gpr1) and a glucose transporter (Hxt1) [3]. CFP- or YFP-tagged forms of these control proteins fail to yield a FRET signal when coexpressed with CFP- or YFP-tagged α-factor receptors. Similarly, overexpression of Gpr1 or Hxt1 from the strong constitutive *PGK* and *ADH* promoters, respectively, on high-copy plasmids does not reduce the apparent efficiency of FRET between CFP- and YFP-tagged α-factor receptors. In contrast, overexpression of untagged α-factor receptors

from the *PGK* promoter on a high-copy plasmid results in complete loss of a FRET signal without affecting expression of the tagged receptors (Fig. 4B).

16. Effects of agonist or antagonist binding on FRET efficiency

A central question in the field is whether GPCR oligomerization is affected on agonist or antagonist binding, as might occur if changes in the oligomerization state are part of the mechanism whereby a GPCR is activated. This question can be addressed by quantifying the efficiency of FRET in the presence and absence of agonist or antagonist. It should, however, be noted that changes in the oligomerization state of GPCRs activated by large peptides or glycoprotein hormones could not be analyzed by performing FRET experiments with intact yeast cells expressing tagged forms of these receptors, because these agonists would be excluded by the yeast cell wall. This limitation can be overcome by performing FRET experiments with purified yeast plasma membrane fractions, as discussed below.

17. FRET experiments with yeast plasma membrane fractions

We have demonstrated the utility of performing FRET experiments with tagged α-factor receptors in purified yeast plasma membrane fractions. These experiments employ standard methods to prepare yeast cell lysates (breaking cells by agitation with glass beads) and plasma membrane fractions (density gradient fractionation) from cells expressing CFP- and/or YFP-tagged α-factor receptors [3]. These FRET experiments use procedures identical to those employed with intact cells as described above except that membrane fractions are used. The results of these experiments have indicated that a similar efficiency of FRET between CFP- and YFP-tagged α-factor receptors is apparent whether plasma membrane fractions or intact cells are used.

18. FRET techniques

Energy transfer has recently been used in a variety of ways to detect both hetero- and homooligomerization of a number of receptors. Techniques have included scanning fluorometry FRET [3], bioluminescense resonance energy transfer (BRET) [32–34] time-resolved FRET [34], photobleaching FRET (pbFRET) microscopy [35–38], spectral imaging microscopy (SPIM) [39], and fluorescence lifetime imaging microscopy (FLIM) [39]. Each of these techniques has certain advantages and disadvantages. BRET is attractive because RLUC luminescence is activated by the substrate coelenterazine, and thus excitation of a fluorescence donor is not required. However, coelenterazine oxidation, autoluminescence, and cell permeability to the substrate may be problematic. Additionally, the sensitivity of BRET can be low due to the high signal-to-noise ratio [40]. Furthermore, a weak BRET signal can be caused by donor and acceptor overexpression, which requires careful monitoring of protein expression [40]. pbFRET, SPIM, and FLIM are all powerful single-cell-based FRET assays. FLIM is advantageous because it allows FRET to be measured directly by monitoring changes in fluorescence lifetimes rather than ratiometric analysis of fluorescence emission intensity [41]. While these techniques are very powerful, they require the ability to perform single-cell FRET microscopy, which is not a technology readily available to most researchers.

19. Concluding remarks

The ease with which scanning fluorometry FRET can be detected between GPCRs expressed in yeast makes it a potentially powerful tool to address several important problems in the field. FRET in yeast provides an attractive means of surveying which GPCRs can self-associate or interact with other GPCRs. Furthermore, evidence of mammalian GPCR oligomerization in yeast would be indicative of a direct interaction, because mammalian cell-specific factors such as PDZ proteins that may bridge interactions between mammalian GPCRs are absent in yeast. This consideration is particularly relevant when conducting structure–function studies aimed at defining the molecular principles that determine the specificity with which GPCRs associate with themselves and/or other GPCRs. Finally, FRET is likely to provide a facile means of identifying peptides or other small molecules that disrupt GPCR oligomerization, thereby providing novel pharmacological tools to examine the importance of GPCR oligomerization in vivo.

Acknowledgments

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References

[1] S.M. Wade, H.M. Dalman, S.Z. Yang, R.R. Neubig, Mol. Pharmacol. 45 (1994) 1191–1197.
[2] T.E. Hebert, S. Moffett, J.P. Morello, T.P. Loisel, D.G. Bichet, C. Barret, M. Bouvler, J. Biol. Chem. 271 (27) (1996) 16384–16392.
[3] M.C. Overton, K.J. Blumer, Curr. Biol. 10 (6) (2000) 341–344.

[4] R.A. Kammerer, S. Frank, T. Schulthess, R. Landwehr, A. Lustig, J. Engel, Biochemistry 38 (40) (1999) 13263–13269.
[5] R. Kuner, G. Kohr, S. Grunewald, G. Eisenhardt, A. Bach, H.C. Kornau, Science 283 (5398) (1999) 74–77.
[6] G.Y. Ng, J. Clark, N. Coulombe, N. Ethier, T.E. Hebert, R. Sullivan, S. Kargman, A. Chateauneuf, N. Tsukamoto, T. McDonald, P. Whiting, E. Mezey, M.P. Johnson, Q. Liu, L.F. Kolakowski Jr., J.F. Evans, T.I. Bonner, G.P. O'Neill, J. Biol. Chem. 274 (12) (1999) 7607–7610.
[7] B.A. Jordan, L.A. Devi, Nature 399 (6737) (1999) 697–700.
[8] R. Maggio, P. Barbier, A. Colelli, F. Salvadori, G. Demontis, G.U. Corsini, J. Pharmacol. Exp. Ther. 291 (1) (1999) 251–257.
[9] Z. Xie, S.P. Lee, B.F. O'Dowd, S.R. George, FEBS Lett. 456 (1) (1999) 63–67.
[10] K. Fuxe, S. Ferre, M. Zoli, L.F. Agnati, Brain Res. Brain Res. Rev. 26 (2-3) (1998) 258–273.
[11] B.A. Jordan, N. Trapaidze, I. Gomes, R. Nivarthi, L.A. Devi, Proc. Natl. Acad. Sci. USA 98 (1) (2001) 343–348.
[12] J.R. Lakowitz, Priciples of Fluorescence Spectroscopy, Plenum, New York, 1983.
[13] T. Okada, I. Le Trong, B.A. Fox, C.A. Behnke, R.E. Stenkamp, K. Palczewski, J. Struct. Biol. 130 (1) (2000) 73–80.
[14] K. Palczewski, T. Kumasaka, T. Hori, C.A. Behnke, H. Motoshima, B.A. Fox, I. Le Trong, D.C. Teller, T. Okada, R.E. Stenkamp, M. Yamamoto, M. Miyano, Science 289 (5480) (2000) 739–745.
[15] A. Miyawaki, J. Llopis, R. Heim, J.M. McCaffery, J.A. Adams, M. Ikura, R.Y. Tsien, Nature 388 (6645) (1997) 882–887.
[16] H.R. Pollok BA, Trends Cell Biol. 9 (1999) 57–60.
[17] A. Miyawaki, R.Y. Tsien, Methods Enzymol. 327 (2000) 472–500.
[18] R. Heim, Methods Enzymol. 302 (1999) 408–423.
[19] I. Erlenbach, E. Kostenis, C. Schmidt, F.F. Hamdan, M.H. Pausch, J. Wess, J. Neurochem. 77 (5) (2001) 1327–1337.
[20] I. Erlenbach, E. Kostenis, C. Schmidt, C. Serradeil-Le Gal, D. Raufaste, M.E. Dumont, M.H. Pausch, J. Wess, J. Biol. Chem. 276 (31) (2001) 29382–29392.
[21] P. Leplatois, A. Josse, M. Guillemot, M. Febvre, N. Vita, P. Ferrara, G. Loison, Eur. J. Biochem. 268 (18) (2001) 4860–4867.
[22] B. Lagane, G. Gaibelet, E. Meilhoc, J.M. Masson, L. Cezanne, A. Lopez, J. Biol. Chem. 275 (43) (2000) 33197–33200.
[23] G. Gaibelet, E. Meilhoc, J. Riond, I. Saves, T. Exner, L. Liaubet, B. Nurnberg, J.M. Masson, L.J. Emorine, Eur. J. Biochem. 261 (2) (1999) 517–523.
[24] T.J. Baranski, P. Herzmark, O. Lichtarge, B.O. Gerber, J. Trueheart, E.C. Meng, T. Iiri, S.P. Sheikh, H.R. Bourne, J. Biol. Chem. 274 (22) (1999) 15757–15765.
[25] L.A. Price, J. Strnad, M.H. Pausch, J.R. Hadcock, Mol. Pharmacol. 50 (4) (1996) 829–837.
[26] L.A. Price, E.M. Kajkowski, J.R. Hadcock, B.A. Ozenberger, M.H. Pausch, Mol. Cell. Biol. 15 (11) (1995) 6188–6195.
[27] S. Arkinstall, M. Edgerton, M. Payton, K. Maundrell, FEBS Lett 375 (3) (1995) 183–187.
[28] K. King, H.G. Dohlman, J. Thorner, M.G. Caron, R.J. Lefkowitz, Science 250 (4977) (1990) 121–123.
[29] P. Payette, F. Gossard, M. Whiteway, M. Dennis, FEBS Lett 266 (1–2) (1990) 21–25.
[30] C.J. Stefan, K.J. Blumer, J. Biol. Chem. 274 (3) (1999) 1835–1841.
[31] J.E. Reneke, K.J. Blumer, W.E. Courchesne, J. Thorner, Cell 55 (2) (1988) 221–234.
[32] S. Angers, A. Salahpour, E. Joly, S. Hilairet, D. Chelsky, M. Dennis, M. Bouvier, Proc. Natl. Acad. Sci. USA 97 (7) (2000) 3684–3689.
[33] K.M. Kroeger, A.C. Hanyaloglu, R.M. Seeber, L.E. Miles, K.A. Eidne, J. Biol. Chem. 276 (16) (2001) 12736–12743.
[34] M. McVey, D. Ramsay, E. Kellett, S. Rees, S. Wilson, A.J. Pope, G. Milligan, J. Biol. Chem. 276 (17) (2001) 14092–14099.
[35] M. Rocheville, D.C. Lange, U. Kumar, R. Sasi, R.C. Patel, Y.C. Patel, J. Biol. Chem. 275 (11) (2000) 7862–7869.
[36] M. Rocheville, D.C. Lange, U. Kumar, S.C. Patel, R.C. Patel, Y.C. Patel, Science 288 (5463) (2000) 154–157.
[37] D.A. Roess, R.D. Horvat, H. Munnelly, B.G. Barisas, Endocrinology 141 (12) (2000) 4518–4523.
[38] R.D. Horvat, D.A. Roess, S.E. Nelson, B.G. Barisas, C.M. Clay, Mol. Endocrinol. 15 (5) (2001) 695–703.
[39] L.G. Tertoolen, C. Blanchetot, G. Jiang, J. Overvoorde, T.W. Gadella Jr., T. Hunter, J. Hertog Jd, BMC Cell. Biol. 2 (1) (2001) 8.
[40] Y. Xu, D.W. Piston, C.H. Johnson, Proc. Natl. Acad. Sci. USA 96 (1) (1999) 151–156.
[41] A.G. Harpur, F.S. Wouters, P.I. Bastiaens, Nat. Biotechnol. 19 (2) (2001) 167–169.